Article

IDENTIFICATION OF A NOVEL BURSICON-REGULATED TRANSCRIPTIONAL REGULATOR, md13379, IN THE HOUSE FLY *Musca domestica*

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Bursicon is a neuropeptide that regulates cuticle sclerotization (hardening and tanning) and wing expansion in insects via a G-protein coupled receptor. The hormone consists of α and β subunits. In the present study, we cloned bursicon α and β genes in the house fly Musca domestica using 3' and 5' RACE and expressed the recombinant bursicon (rbursicon) heterodimer in mammalian 293 cells and insect Highfive™ cells. The rbursicon displayed a strong bursicon activity in the neck-ligated house fly assay. Using rbursicon, we identified and cloned a novel bursicon-regulated gene in M. domestica encoding a transcriptional regulator homologous to ataxin-7-like3 in human, CG13379 in Drosophila and sgf11 in yeast Saccharomyces cerevisiae. We named the gene md13379. Both ataxin-7-like3 and sgf11 are a novel subunit of the SAGA (Spt-Ada-Gcn5-Acetyltransferase) complex that is involved in regulation of gene transcription. Real-time PCR analysis of temporal

Abbreviations: rbursicon, recombinant bursicon; RACE, rapid amplification of cDNA ends; SAGA, Spt-Ada-Gcn5-Acetyltransferase; CNS, central nervous system

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response profile revealed that the level of md13379 transcript was up-regulated by 6.6 fold 1 h after rbursicon injection, which correlates well with the cuticle sclerotization process observed in the rbursicon-injected flies. The composite data suggest that md13379 plays a role in regulating the expression of bursicon-regulated genes involved in the cuticle sclerotization process. © 2008 Wiley Periodicals, Inc.

Keywords: recombinant bursicon; Sgf11; real-time PCR; functional analysis; cuticle sclerotization

INTRODUCTION

Bursicon is a neuropeptide responsible for cuticle sclerotization (hardening and tanning) and wing expansion in insects. It was first described over four decades ago as a bioactive cuticle sclerotization factor present in the central nervous system (CNS) and hemolymph of newly emerged adults in a neck-ligated blow fly assay (Fraenkel & Hsiao, 1962, 1965; Cottrell, 1962a). Since its discovery, bursicon had been proposed to be a single protein with a molecular size between 30 and 60 kDa, which varies greatly among insect species. However, recent studies have demonstrated that in *Drosophila melanogaster*, bursicon is a heterodimer protein consisting of two subunits named bursicon α (AJ862523) and bursicon β (AJ862524) with molecular weights of 16 kDa and 14 kDa respectively (Luo et al., 2005; Mendive et al., 2005). Bursicon is the first heterodimeric cystine knot hormone discovered in insects (Luo et al., 2005; Mendive et al., 2005). Based on available gene sequences at NCBI data bank, both *Drosophila* bursicon α and β have similar sequence matches in five insect species including *Anopheles gambiae, Apis mellifera, Bombyx mori, Manduca sexta* and *Tribolium castaneu*, and two crustacean species, *Carcinus maenas and Daphnia arenata*.

Bursicon is the natural ligand of a G protein-coupled receptor, DLGR2, which is encoded by the *rickets* gene (Baker and Truman, 2002). Upon activation, DLGR2 is hypothesized to activate the cAMP/PKA signaling pathway (Kimura et al., 2004). Recombinant bursicon (rbursicon) heterodimer also has a high-affinity binding to DLGR2 and initiates cuticle tanning in the ligated fruit fly bioassay (Luo et al., 2005; Mendive et al., 2005). In fruit flies, mutation of either bursicon α gene or receptor *rickets* gene causes defects in cuticle sclerotization and wing expansion (Baker and Truman, 2002; Dewey et al., 2004). Recent studies have shown that tyrosine hydroxylase, the enzyme mediating the conversion of tyrosine to a tanning agent precursor DOPA, is activated by bursicon via DLGR2 and PKA (Davis et al., 2007), leading to transcription of a set of genes required for cuticle sclerotization (An et al., 2008). A gene silencing study revealed that injection of double-stranded bursicon α RNA into *B. mori* pupae significantly reduced the level of bursicon α mRNA in pupae, resulting in a decrease in wing expansion (Huang et al., 2007). Although studies of bursicon have made great progress, signal transduction components downstream of bursicon receptor DLGR2 and the roles of bursicon regulated genes in cuticle sclerotization processes remain unclear.

In the present study, we report the molecular cloning, recombinant expression of *Musca domestica* bursicon. We also report identification of a novel bursicon-regulated house fly gene.

MATERIALS AND METHODS

Experimental Insects

House fly larvae were reared on artificial diet (Carolina Biological Supply Company, Burlington, NC) at 30°C under constant darkness and adults were fed on a 1:1 mixture of granulated sugar and powder milk at 30°C under 16 h L: 8 h D.

Cloning of Bursicon α and β Genes and md13379 Using 5' and 3' RACE

To obtain the full-length house fly bursicon (α and β) and md13379 cDNAs, rapid amplification of cDNA ends (RACE) was performed using a 5'RACE and 3'RACE system (Invitrogen, Carlsbad, CA). The RACE primers (Table 1) were designed based on the conserved bursicon sequences of *D. melanogaster*, *T. castaneum*, *A. gambiae*, *B. mori*, and *A. mellifera*.

The house fly CNS (the fused thoracic and abdominal ganglia without brain) were dissected under Ringers' solution (3.6 mM NaCl, 54.3 mM KCl, 8.0 mM $CaCl_2$, and 28.3 mM $MgCl_2$) from pharate adults (n = 10 adults). Total RNA was extracted from the CNS using Trizol reagent (Invitrogen) according to the manufacturer's instructions. For the amplification of 3' ends of cDNA, the first-strand cDNA was synthesized using the manufacturer-supplied adapter primer. The supplied abridged universal amplification primer, the gene specific primer and the nested primer were used in subsequent amplification. For the amplification of 5' ends of cDNA, the gene specific primer was used for the first-strand cDNA synthesis. Two nested primers were used for subsequent amplification. The PCR products from 3' and 5' RACE were purified, sequenced at the MU DNA Core Facility, and used as references for designing gene specific primers.

Table 1. Gene Specific Primers (GSPs) for Bursicon α and β and for a Transcriptional Regulator md13379 in 3′ and 5′ RACE

Bursicon α	3′-RACE		
	Primer 1:	5′-AAGATCTGGCAAATGGACCG-3′	(GSP)
	Primer 2:	5′-CCTGCATGTGCTGCCAGGA-3′	(Nested GSP)
	5′-RACE		
	Primer 1:	5′-GCATGGCCGACACATGCACT-3′	(GSP)
	Primer 2:	5′-TCCTGGCAGCACATGCAGG-3′	(Nested GSP)
	Primer 3:	5′-CGGTCCATTTGCCAGATCTT-3′	(Nested GSP)
Bursicon β	3′-RACE		
	Primer 1:	5′-TGCAACAGTCAGGTGCAACC-3′	(GSP)
	Primer 2:	5′-GAAAGACTGCTACTGCTGCCG-3′	(Nested GSP)
	5′-RACE		
	Primer 1:	5′-ATCGCCACATTTGAAGCACT-3′	(GSP)
	Primer 2:	5′-CGGCAGCAGTAGCAGTCTTTC-3′	(Nested GSP)
	Primer 3:	5′-GGTTGCACCTGACTGTTGCA-3′	(Nested GSP)
md13379	3′-RACE		
	Primer 1:	5′- AGGCTGCCAACTATTTGTAT-3′	(GSP)
	Primer 2:	5′- TCGCATTGTTGACATGCCGA-3′	(Nested GSP)
	5′-RACE		
	Primer 1:	5′- CCCATACCCATGCACTTTTC-3′	(GSP)
	Primer 2:	5′- TGGGGCAGGTGCAGTCCATTGGT-3′	(Nested GSP)
	Primer 3:	5′- TCTTAGCGGTGGATATGCCAA-3′	(Nested GSP)

Table 2. Primers for PCR Amplification of Full Length Bursicon α and β cDNA.

Bursicon α forward primer:	5′-ACTCGAGATGGAAGTTTCAGTTTTTCG-3′;
Bursicon β forward primer:	5′-ACTCGAGATGCTTAAATTGTGGAAATT-3′;
Bursicon α reverse primer:	5′-AGGATCCCTGCAATGCTATCCTTCTG-3′;
Bursicon β reverse primer:	5′-AGGATCCTCTCGTGAAATCACCACAT-3′

The underlined indicates the inserted *Xho*I restriction site (CTCGAG) for forward primers and *BamH*1 restriction site (GGATCC) for reverse primers.

Protein Sequence Alignments

Analyses of the deduced amino acid sequences of the house fly bursicon (α and β) and md13379 were carried out using the ExPASy server (http://www.ca.expasy.ch) for prediction of domains, motifs, and signal peptides. Homology searches of the house fly bursicon (α and β) and md13379 proteins were made on NCBI platform (http://www.ncbi.nih.gov). Alignments of multiple sequences were carried out with ClustalW and edited with Genedoc software.

Analysis of Intron-Exon Arrangement of the House Fly Bursicon α and β Genes

To obtain the introns of the house fly bursicon α and β genes, PCR was carried out using the house fly genome DNA as a template. For amplification of bursicon α gene, primers 5'-TCGGTGTAATAATCAAAATCTG-3' and 5'-TGGGTGGTCCTTCATCC GAAT-3'were used. The PCR fragment was inserted into the PGEM-T-Easy vector (Promega, Madison, WI) and further sequenced. Similarly, primers 5'-ATGCTT AAATTGTGGAAATTA-3' and 5'-TTATCTCGTGAAATCACCACA-3'were used to amplify bursicon β gene. Intron-exon arrangement of bursicon α and β genes among six insect species was aligned and analyzed.

The cDNAs and DNA sequences of bursicon α and β genes were aligned with each other for the identification of introns.

Expression of Recombinant Bursicon in Mammalian and Insect Cells

To express recombinant house fly bursicon α and β heterodimer protein, the open reading frames of bursicon α and β were first amplified using the forward primers with *Xho*I restriction site (CTCGAG) and reverse primers with *BamH*1 restriction site (GGATCC) (Table 2), cloned into PGEM-T-Easy vector (Promega), and sequenced again for confirmation of correct insertion.

To express the recombinant house fly bursicon in mammalian 293 cells, bursicon α and β cDNAs were retrieved from the PGEM-T-Easy vector using *Xho*I and *BamH*1 and ligated respectively into pcDNA3.1 expression vector predigested with *Xho*I and *BamH*1. The pcDNA3.1 vector containing bursicon α or β cDNA was further sequenced for confirmation of correct insertion.

The pcDNA3.1 plasmid (2 μg) containing bursicon α or β was used to transfect mammalian 293 cells either individually or simultaneously using SatisFection™ Transfection Reagent (Stratagene, La Jolla, CA). After 16 h initial transfection, the serum-free DMEM cell culture medium was replaced with the same medium containing 10% fetal bovine serum and the transfected cells were incubated for additional 24 h. The cell medium was then replaced with serum-free DMEM medium and cultured for 48 h. At the end of incubation, the medium was collected, centrifuged at 2000 × g to remove cell debris and stored at −80°C for bursicon bioassay.

Bursicon α and β recombinant protein was also expressed in insect High Five™ cells using Bac-to-Bac baculovirus expression system according to the manufacturer's protocol (catalog ♯10359, Invitrogen). In brief, bursicon α and β cDNAs, retrieved from the PGEM-T-Easy vector using *Xho*I and *BamH*1, were ligated into pFastBac™ donor plasmids respectively. The purified pFastBac™ constructs were used to transform DH10Bac™ *E. coli* cells to generate recombinant bacmid. The recombinant bacmid DNA was analyzed using PCR for confirmation of recombination and used to transfect insect High Five™ cells to produce recombinant baculovirus. The recombinant baculoviruses were used to infect insect High Five™ cells to express the rbursicon protein. The expressed rbursicon protein was analyzed using Western blot before bursicon bioassay to ensure the rbursicon expression.

Bioassay of Recombinant Bursicon

The rbursicon was assessed for its hormonal activity in the house fly adults neck-ligated with dental floss immediately after emergence. After 1 h, 0.5 µl (120 ng/µl) of the recombinant protein containing bursicon α or bursicon β (control) or the bursicon α and β heterodimer was injected into the thorax-abdomens of untanned ligated flies using a glass needle mounted on a microinjection system. The medium, which had been passed over the Ni-NTA His bind resin (Qiagen, Valencia, CA) from mammalian 293 cells transfected with blank pcDNA3.1 plasmid or from insect High Five™ cells transfected with blank bacmid, was used as a sham control. The purified rbursicon protein was quantified using a protein quantification kit (Catalog ♯500–0113 and ♯500–0114, Bio-Rad, Hercules, CA). The CNS extract of newly emerged flies was used as a positive control (0.5 CNS equivalent/fly). Cuticle sclerotization was evaluated at 3 h after injection and photographed using a Leica MZ16 microscope with a Q-imaging digital camera and MicroPublisher 5.0 software.

Real-Time PCR Analysis of Bursicon-Regulated md13379 Gene

To analyze bursicon regulated genes, newly emerged house fly adults were neck-ligated immediately after emergence. After one hour, the neck-ligated flies that did not show any cuticle tanning were injected with rbursicon α and β heterodimer protein using a microinjection system. Bursicon α dissolved in the same injection buffer was used as negative control. Injected flies were collected at the indicated time periods after injection (20 min, 40 min, 1 h, 2 h and 12 h) and placed at −80°C for subsequent experiments.

Total RNA was isolated from the flies using Trizol reagent and treated with RNase-free DNase I. RNA concentration was determined by measuring the absorbance at 260 nm on a spectrophotometer. Total RNA (2 µg) from each sample was used for the first-strand cDNA synthesis as described previously (Wang et al., 2008). The synthesized cDNA was used as a template for analysis of gene transcription by real-time PCR. Primers designed for *md13379* were: 5'-AGGCTGCCAACTATTTGTAT-3' (forward) and 5'-CCCATACCCATGCACTTTTC-3' (reverse). RP49 gene (forward primer: 5'-TAC AGGCCCAAGATCGTGAA-3'; reverse primer: 5'-GACAATCTCCTTGCGCTTCT-3') was used for normalization. Real-time PCR amplification and analysis were carried out on an Applied Biosystems 7300 Fast Real-Time PCR System (ABI). The final reaction volume was 20 µl using ABI SYBR Green Supermix (ABI). The thermal cycle conditions used in real time PCR were: 95°C for 10 min, followed by 40 cycles of 95°C for 15 sec and 60°C for 1 min. The specificity of SYBR green PCR signal was confirmed by the melting curve analysis and agarose gel electrophoresis. The mRNA expression was quantified

using the comparative CT (Cross Threshold, the PCR cycle number that crosses the signal threshold) method (Livak & Schmittgen, 2001). The CT of the gene RP49 was subtracted from CT of the target gene to obtain ΔCT. The normalized fold changes of the target gene mRNA expression were expressed as $2^{-\Delta\Delta CT}$, where ΔΔCT is equal to $\Delta CT_{\text{treated sample}} - \Delta CT_{\text{control}}$.

Temporal Analysis of md13379 Transcript in Different Tissues

In order to analyze the temporal profile of md13379 transcript in different tissues, the newly emerged house fly adults were neck-ligated immediately after emergence. After one hour, the neck-ligated flies that did not show any cuticle tanning were injected with rbursicon protein using a microinjection system. Specific tissues (epidermis, central nervous system, midgut, fat body) were collected from the injected flies at the indicated time periods after injection (0 min, 20 min, 40 min, 1 h, 2 h, and 12 h) and placed at −80°C for subsequent experiments.

Total RNA extraction, cDNA synthesis, and real-time PCR analysis of md13379 gene in different tissues were carried out according to the protocol described above.

RESULTS

Cloning and Sequence Analysis of Bursicon α and β Genes

Full length sequences of the house fly bursicon α and β genes were obtained using 3' and 5' RACE. As shown in Fig. 1, the house fly bursicon α gene has an ORF of 531 bps, encoding a 176 amino acid peptide. The polyadenylation signal sequence (AATAAA) is localized 99 nts downstream of the stop codon (TAA) (Fig. 1A). The bursicon β gene has an ORF of 444 bps, encoding a 147 amino acid peptide. The polyadenylation signal sequence is localized 75 nts downstream of the stop codon (Fig. 1B).

Multiple Protein Sequence Alignments

The ExPASy search results revealed a C-terminal cystine knot domain (a.a. 55–145) and a cell attachment sequence (a.a. 36–38) in the house fly bursicon α sequence (Fig. 2A). The bursicon β subunit has an N-glycosylation site (a.a. 22–25), two N-myristoylation sites (a.a. 73–78 and a.a.116–121), and a C-terminal cystine knot domain (a.a. 45–143) (Fig. 2B).

Sequence alignment revealed that the house fly bursicon α shares a 79% sequence identity with *D. melanogaster*, 61% with *T. castaneum*, 56% with *A. mellifera*, 55% with *A. gambiae*, 49% with *B. mori,* 48% with *M. sexta*, 47% with *D. arenata* (Wilcockson and Webster, 2008), and 42% with *Carcinus maenas* respectively (Fig. 2A). Similarly, the house fly bursicon β has a 79% sequence identity with *D. melanogaster*, 55% with *T. castaneum*, 51% with *B. mori*, 50% with *A. mellifera,* 50% with *M. sexta,* 47% with *A. gambiae*, 44% with *D. arenata* and 46% with *C. maenas* respectively (Fig. 2B). Significant variation occurs at N-terminal regions of bursicon α and β among the nine species analyzed. However, if the signal peptide at the N-terminal is excluded from the sequences of the nine species (signal peptide splitting site is indicated by arrow), the mature housefly bursicon α and β subunits share a much higher sequence identity with other species, up to 92% and 93% with *Drosophila* counterparts, respectively. The cystine knot sequence in bursicon α and β subunits is highly conserved. The house fly bursicon α shares a 98% identity with the *Drosophila* sequence in this region and a 71%

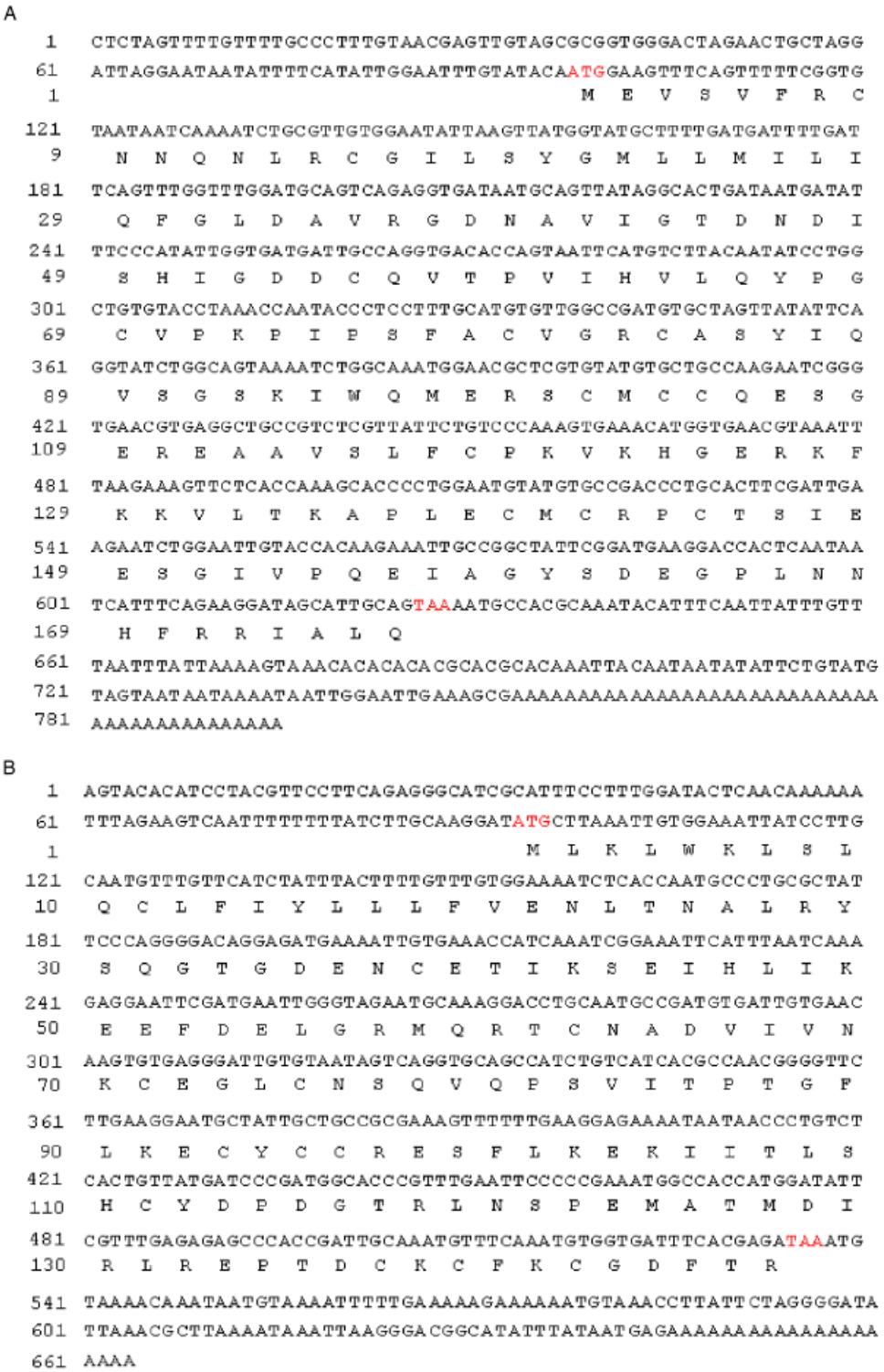

Figure 1. The house fly bursicon nucleotide acid and predicted amino acid sequences. A: Bursicon α; B: Bursicon β.

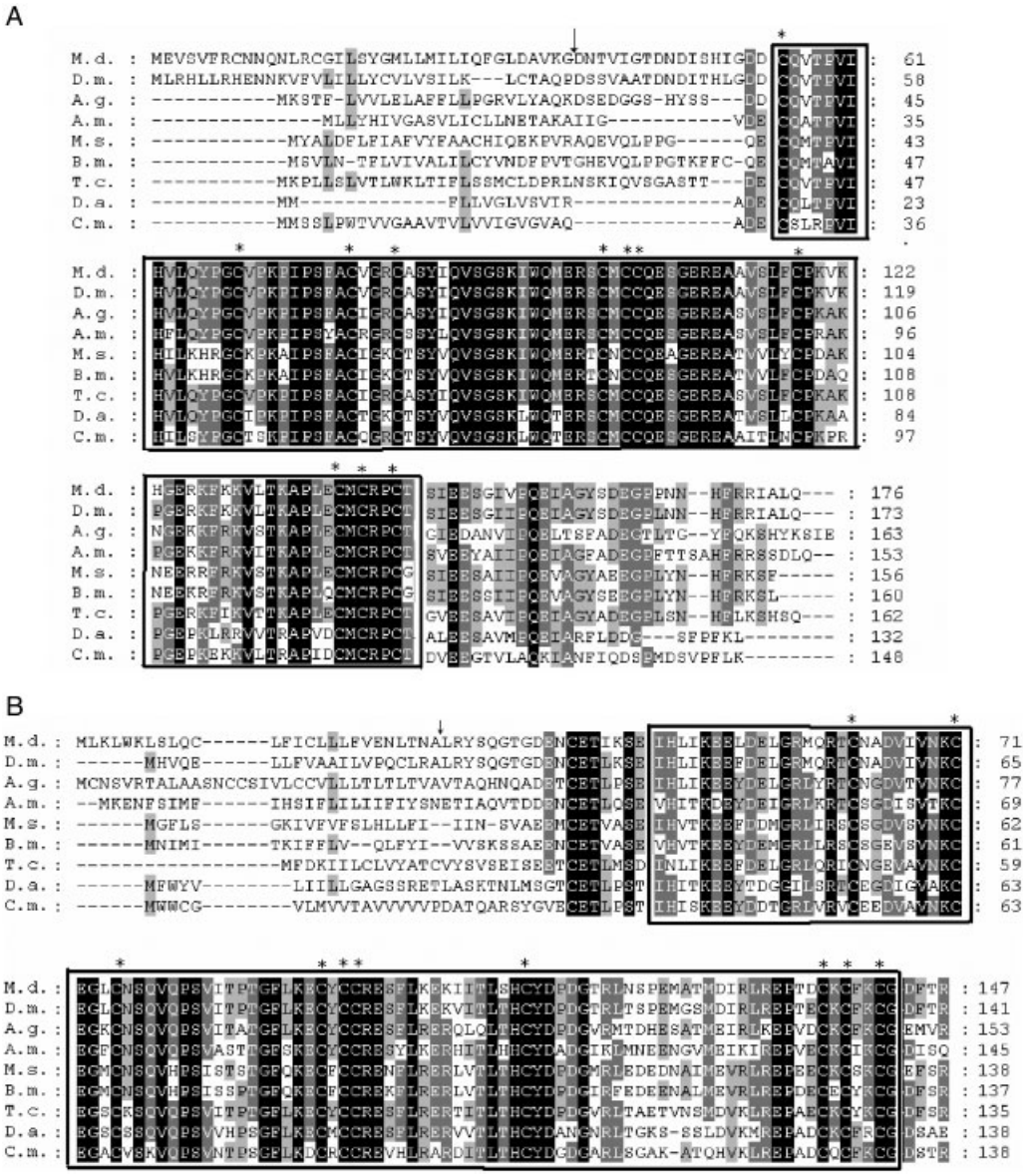

Figure 2. Alignments of the house fly bursicon α (A) and β (B) amino acid sequences with the sequences from other species. A.g.: *Anopheles gambiae*. A.m.: *Apis mellifera*. B.m.: *Bombyx mori*. C.m.: *Carcinus maenas*. D.a.: *Daphnia arenata*. D.m.: *Drosophila melanogaster*. M.d.: *Musca domestica*. M.s.: *Manduca sexta*. T.c.: *Tribolium castaneum*. The dark shaded residues indicate 100% identity and the gray shading represents 80% identity between analyzed species. The box represents cystine knot region. The asterisk shows the conserved cysteine residues and the arrow indicates the signal peptide cleavage site.

to 95% identity with sequences of the rest insect species (Fig. 2A). The similar is true for bursicon β (Fig. 2B). Most importantly, the 10 cysteine residues in bursicon α and β subunits are all conserved in the species analyzed (Fig. 2A,B).

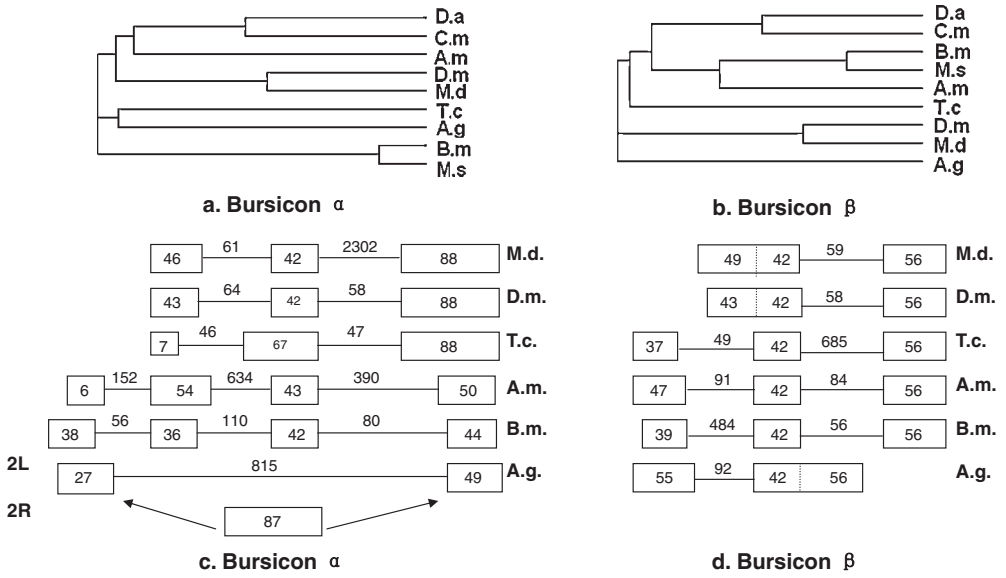

Figure 3. Phylogenic analysis of bursicon α (A) and β (B) and gene structure analysis of bursicon α (C) and β (D) in the house fly and other species.

Phylogenetic and Gene Structure Analysis of Aursicon α and β Subunits

Phylogenetic analysis of bursicon α and β showed that *Musca* bursicon was most closely related to *Drosophila* counterparts (Fig. 3A,B). Molecular cloning and gene sequence analysis revealed that *Musca* bursicon α gene consists of three coding exons and two introns, identical to that in *D. melanogaster*, *T. castaneum* and *A. gambiae*, but different from that in *A. mellifera* and *B. mori,* each with four coding exons and three introns (Fig. 3C) (*Manduca* bursicon gene structure is not available from GenBank, therefore is not included in the analysis). The exon 2 in *M. domestica* and *D. melanogaster* is only a portion of the exon 2 in *T. castaneum*. All analyzed bursicon α genes encoding exons are located at the same left chromosome arm except the exon 2 in *A. gambiae* which is at the right chromosome arm.

Similarly, bursicon β gene consists of two coding exons in *M. domestica*, *D. melanogaster*, and *A. gambiae* or three coding exons in *A. mellifera, B. mori*, and *T. castaneum* (Fig. 3D). The exon 1 in *M. domestic* and *D. melanogaster* are divided into two exons (exon 1 and 2) in *A. mellifera, B. mori*, and *T. Castaneum*, whereas the exon 2 and 3 in *A. mellifera B. mori*, and *T. castaneum* are merged into a single exon (exon 2) in *A. gambiae*.

Functional Analysis of the Rbursicon in Neck-Ligated Flies

The rbursicon protein, expressed in mammalian 293 cells or insect Highfive™ cells, was assayed for bursicon activity in the neck-ligated house fly assay. As shown in Fig. 4, no sign of cuticle tanning was observed in the neck-ligated flies injected with 0.5 µl of the supernatant from cell culture transfected with blank vector only (Fig. 4A) or vector with bursicon α (Fig. 4B) or vector with bursicon β (Fig. 4C). Cuticle tanning was detected only in the neck-ligated flies injected with the CNS homogenate (0.5 CNS equivalent) (Fig. 4D) or the supernatant of insect Highfive™ cells (Fig. 4E) and

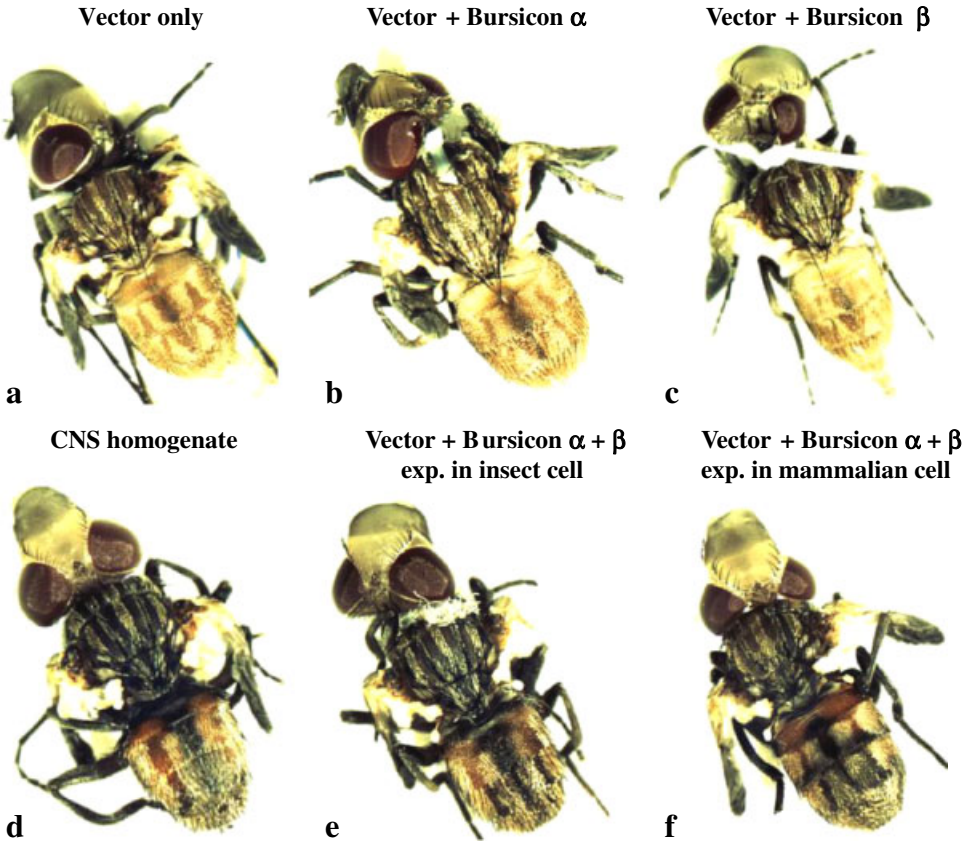

Figure 4. Functional assay of the recombinant bursicon heterodimer in neck-ligated house flies. Newly emerged flies were neck-ligated immediately after emergence. The neck-ligated flies with the unsclerotized cuticle 1 h after neck-ligation were individually injected with 0.5 µl of cell culture transfected with blank pcDNA 3.1 vector as negative control (A) or the vector with bursicon α (B) or the vector with bursicon β (C) or the bursicon α vector and the bursicon β vector expressed in insect High Five™ cells (E) and in mammalian 293 cells (F). CNS homogenate (0.5 CNS equivalent) from newly emerged flies was used as positive control (D). Cuticle sclerotization was photographed 3 h after injection.

mammalian 293 cells (Fig. 4F) co-transfected with burcison α and β vectors. An identical result was obtained when the neck-ligated house fly was injected with the *Drosophila* rbursicon heterodimer expressed in mammalian 293 cells and insect Highfive™ cells (data not shown).

Sequence analysis and alignment of md13379

Using 3' and 5' RACE, a full length sequence of a novel bursicon-regulated gene homologous to *Drosophila* CG13379 was obtained and named *md13379*. The md13379 gene has an ORF of 660 bps, encoding a 220 a.a. polypeptide (Fig. 5). Biologically significant domain analysis revealed a motif in md13379 amino acid sequence (98–152 a.a.) that is homologuous to ataxin-7-like3 in *Homo sapiens*, CG13379 in *Drosophila*, and sgf11 in the yeast *Saccharomyces cerevisiae*. These homologs were identified as a novel subunit of the SAGA (Spt-Ada-Gcn5-Acetyltransferase) complex that possesses histone

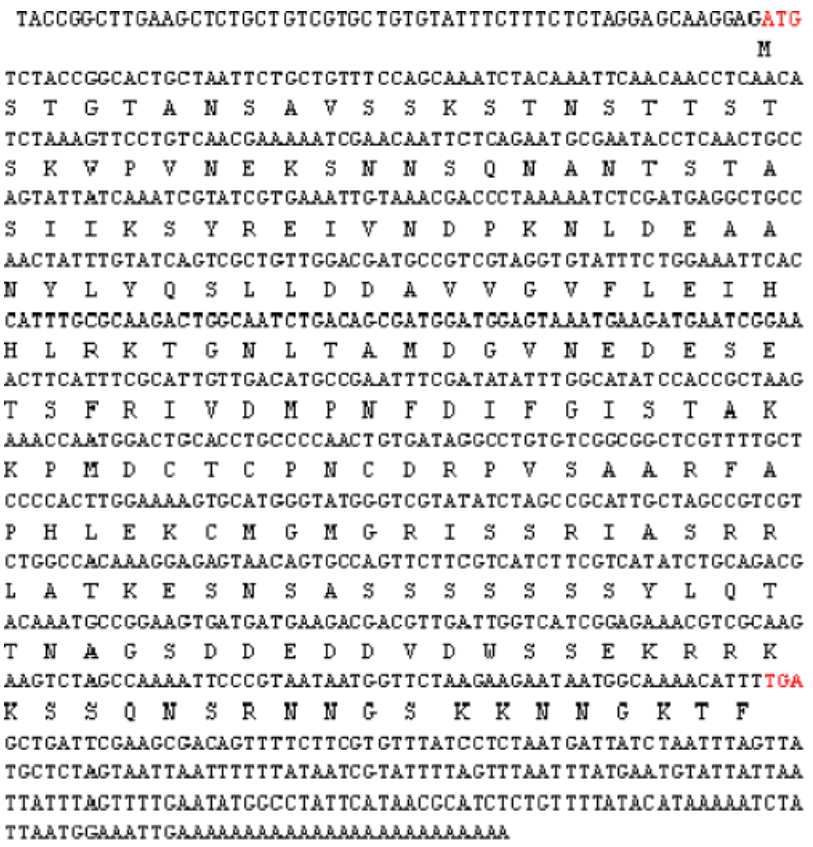

Figure 5. Nucleotide and putative amino acid sequence of md13379.

acetyltransferase activity and is involved in regulation of gene transcription. Sequence alignment of *md13379* and other species including human and yeast revealed that there is a highly conserved Cys and His forming a Cys-X2-Cys-X11-His –X3-4-His/Cys motif (Fig. 6).

Real-Time PCR Analysis of md13379 Transcript after Bursicon Stimulation

As shown in Fig. 7, the level of *md13379* transcript in the rbursicon-injected flies increased at 20 min after bursicon stimulation, reached the maximum of 6.6-fold at 1 h, and dropped sharply thereafter to below basal level at 12 h compared to control (injected with rbursicon α only). A similar result was also obtained in *D. melanogaster* that the *cg13379* transcript was up-regulated by rbursicon in the neck-ligated *Drosophila* fly assay (data not shown), thus confirming the above result.

Temporal Analysis of md13379 Transcript in Different Tissues

Temporal analysis of md13379 transcript in different tissues revealed that the *md13379* transcript was up-regulated by rbursicon only in CNS while there was an apparent inhibitory effect in epidermis, fat body and midgut (Fig. 8). The level of *md13379* in CNS started to increase 20 min after rbursicon injection, reached the maximum value at 1 h, and declined sharply at 2 h.

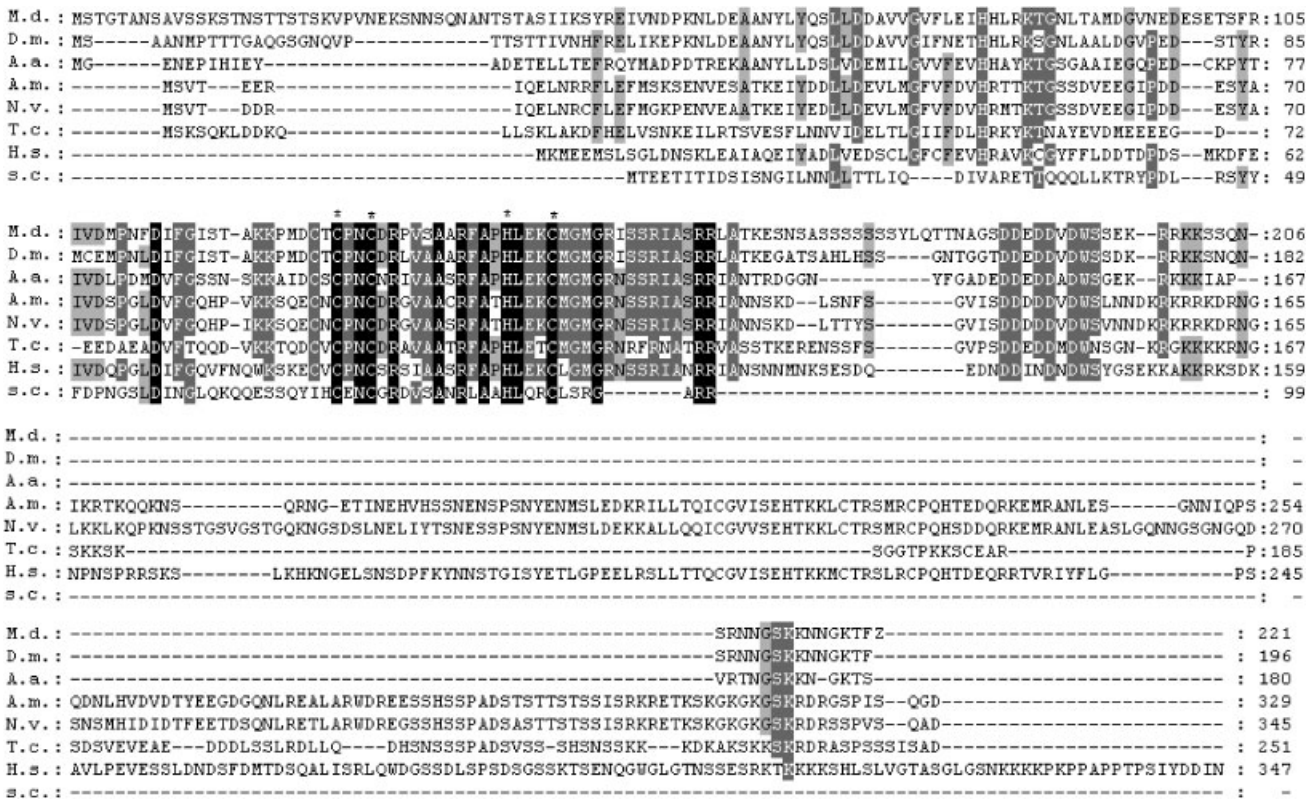

Figure 6. Alignments of the house fly md13379 protein sequences with its homologues. A.a. *Aedes aegypti*: A.m: Apis mellifera. D.m.: Drosophila melanogaster. M.d.: Musca domestica, T.c.: *Tribolium castaneum*, N.v.: *VNasonia vitripennis*, H.S.: Homo sapiens, S.c.: *Saccharomyces cerevisiae*. The dark shaded residues indicate 100% identity and the gray shading represents 80% identity between analyzed species and the asterisk shows the conserved cysteine and histine residues.

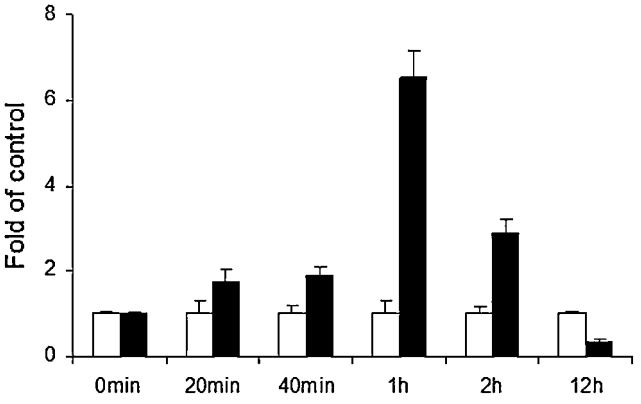

Figure 7. Real-time PCR analysis of the transcriptional expression of md13379 in *M. domestica*. The newly emerged flies were neck-lighted immediately after emergence and injected with 0.5 µl of the recombinant bursicon heterodimer (100 ng/µl). The control received 0.5 µl bursicon α only (100 ng/µl). The RNA was isolated for real-time PCR analysis from the flies at the indicated time periods after bursicon injection. The data represent the mean ± SE of three biological samples. The blank bars represent the samples injected with only bursicon α (control). The black bars represent the samples injected with the recombinant bursicon heterodimer (treatment).

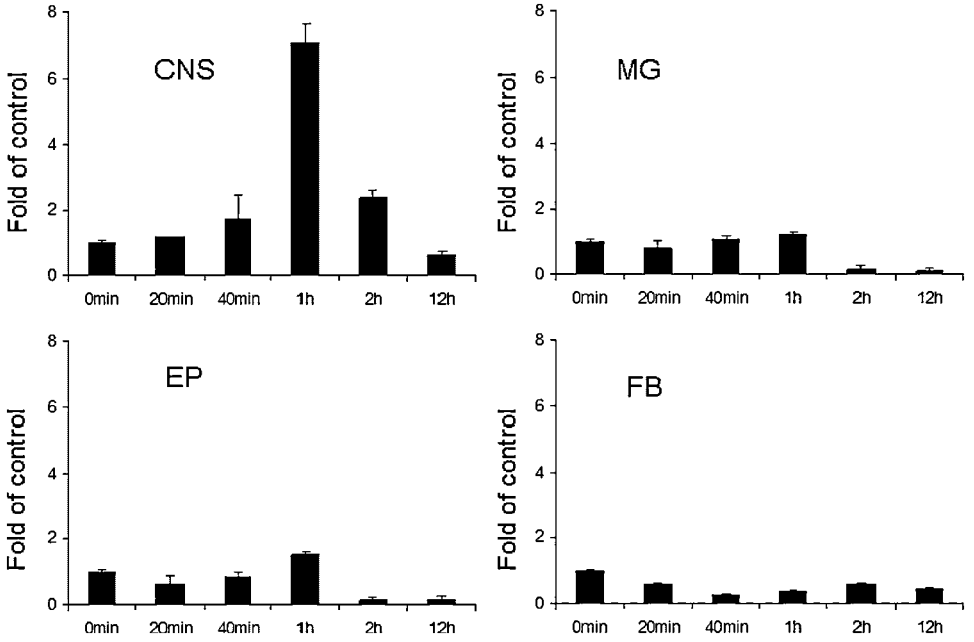

Figure 8. Real-time PCR analysis of the transcriptional expression of md13379 in different tissues (epidermis, nervous system, midgut, fat body) of *M. domestica*. The total RNA from epidermis, nervous system, midgut, and fat body were collected at indicated time periods after injection (0 min, 20 min, 40 min, 1 h, 2 h, and 12 h). The data represent the mean ± SE of three biological samples.

DISCUSSION

For this report, we cloned bursicon α and β cDNAs from the house fly *M. domestica* using 3' and 5' RACE and expressed the recombinant house fly bursicon heterodimer

in both mammalian 293 and insect Highfive™ cells. Our results showed that the rbursicon exerts strong biological activity in the neck-ligated fly assay. Sequence alignment revealed that the house fly bursicon α and β subunits share a 79% sequence identity with the *Drosophila* counterparts. The sequence identity goes up to 92% to 93% when the signal peptides from the N-terminal are excluded from both species. This prompted us to test whether the rbursicon from *Musca* has a cross-species activity in the *Drosophila* fly assay. As expected, the *Musca* rbursicon exhibited a strong tanning activity in the *Drosophila* and vice versa (data not shown). This is not a surprise since the brain extract or hemolymph from several dipteran species including *Sarcophaga bullata* (Baker and Truman 2002; Cottrell, 1962b; Fogal and Fraenkel, 1969), *Phormia regina* (Fraenkel and Hsiao, 1965), and *Lucilia spp* (Cottrell, 1962b; Seligman and Doy, 1972) induce cuticle sclerotization in a blowfly assay. It is reasonable to assume that the genes regulated by bursicon in *M. domestica* would be similar, if not identical, to those in *D. melanogaster*. The genes regulated by rbursicon at ≥ 2 fold in *D. melanogaster* have been reported in our most recent microarray analysis (An et al., 2008). However, the genes up- or down-regulated by rbursicon ≥ 1.5 fold, but ≤ 2 fold in microarray analysis, might also be potential targets of bursicon. Thus, we selected a novel bursicon-regulated gene, CG13379, which was up-regulated by rbursicon by 1.6 fold in *Drosophila* microarray analysis, for further study in *M. domestica*.

Molecular cloning and sequence analysis revealed that the md13379 gene encodes a transcriptional domain. Homology searches of the house fly md13379 proteins revealed that the md13379 is a homologue of ataxin-7-like3 in human, CG13379 in *Drosophila*, and sgf11 in the yeast *S. cerevisiae*. Both ataxin-7-like3 in human and sgf11 in yeast have been identified as novel subunits of a SAGA complex. The SAGA complex is a multi-subunit protein complex consisting of more than 20 proteins and is involved in histone acetylation and transcriptional regulation (Ingvarsdottir et al., 2005). During the process of gene transcription, histone modifications need multi-component protein complexes to perform. For example, SAGA complex acts to acetylate primarily histones H3 and H2B and deubiquitylate histone H2B. As a transcriptional regulator, SAGA plays a vital role in hormone-regulated gene transcription. For example, in *Drosophila*, SAGA is required for expression of ecdysone-response genes, including *broad*, an early-ecdysone response transcription factor (Weake et al., 2008).

In our real-time PCR analysis, the level of *md13379* transcript increased shortly after bursicon stimulation, reached the maximum of 6.6 folds at 1 h, and declined sharply at 2 h. The fluctuation in the level of *md13379* transcript correlates with the bursicon-induced cuticle sclerotization, in which the cuticle tanning in the rbursicon-injected flies was noticed 1 h after rbursicon injection, increased cuticle darkening at 2 h, and a fully tanned cuticle was reached at about 3 h. These results suggest to us, but certainly do not prove, that *md13379* is essential for bursicon-regulated gene expression via its role as a subunit of SAGA complex.

Tissue-specific analysis of *md13379* transcript profile revealed that it is up-regulated mainly in the CNS, but not tested in other tissues. In insects that undergo a complete metamorphosis, the CNS undergoes a drastic remodeling, which includes the differentiation of adult-specific neurons, the coordinated death of some larval neurons, and the remodeling of others (Williams and Truman, 2002). We speculate that the product of the md13379 gene may play a yet-to-be identified role in the CNS immediately after molting from pupae to adult to complete the remodeling process for its full function in adult stage (Williams and Truman, 2002).

Sclerotization is a complex process, in which many genes are activated or repressed after release of bursicon. Gene transcriptional regulation involves covalent chemical modifications of the core histone protein, including acetylation, methylation and phosphorylation. These modifications contribute to on/off switches for gene regulation. Our results demonstrate that *md13379*, a putative transcriptional regulator in the house fly, is up-regulated by rbursicon. Study is underway to reveal the nature of the linkage between bursicon, *md13379*, and downstream-regulated genes involved in the cuticle sclerotization process.

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